



## N-Glycoprotein biosynthesis in plants: recent developments and future trends

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### Abstract

N-glycosylation is a major modification of proteins in plant cells. This process starts in the endoplasmic reticulum by the co-translational transfer of a precursor oligosaccharide to specific asparagine residues of the nascent polypeptide chain. Processing of this oligosaccharide into high-mannose-type, paucimannosidic-type, hybrid-type or complex-type N-glycans occurs in the secretory pathway as the glycoprotein moves from the endoplasmic reticulum to its final destination. At the end of their maturation, some plant N-glycans have typical structures that differ from those found in their mammalian counterpart by the absence of sialic acid and the presence of  $\beta(1,2)$ -xylose and  $\alpha(1,3)$ -fucose residues. Glycosidases and glycosyltransferases that respectively catalyse the stepwise trimming and addition of sugar residues are generally considered as working in a co-ordinated and highly ordered fashion to form mature N-glycans. On the basis of this assembly line concept, fast progress is currently made by using N-linked glycan structures as milestones of the intracellular transport of proteins along the plant secretory pathway. Further developments of this approach will need to more precisely define the topological distribution of glycosyltransferases within a plant Golgi stack. In contrast with their acknowledged role in the targeting of lysosomal hydrolases in mammalian cells, N-glycans have no specific function in the transport of glycoproteins into the plant vacuole. However, the presence of N-glycans, regardless of their structures, is necessary for an efficient secretion of plant glycoproteins. In the biotechnology field, transgenic plants are rapidly emerging as an important system for the production of recombinant glycoproteins intended for therapeutic purposes, which is a strong motivation to speed up research in plant glycobiology. In this regard, the potential and limits of plant cells as a factory for the production of mammalian glycoproteins will be illustrated.

### Introduction

After a decade of relatively low activity, plant glycobiology looks reactivated through the introduction of new technologies and the boost of economic perspectives offered by the use of plants as factories to produce, for a low cost, recombinant proteins that can be used for human therapy [13]. From the boring descriptions of similar or identical structures proposed to have the limited roles of folding helpers or protection from proteolytic degradation of the polypeptides backbone, we are now entering a new area where the

diversity, the originality of plant glycan structures and their functions are rapidly emerging.

In this review, we will point out what is known in plant glycobiology, particularly what has been recently shown concerning N-linked glycoproteins in plants.

*An ever growing structural diversity: from classical PHA-type to Lewis  $\alpha$ -containing plant N-glycans*

In plants, as in other eukaryotes, N-glycans are covalently linked to specific Asn residues constitutive of N-glycosylation sites of the protein. The N-glycosylation

sites are the tripeptide Asn-X-Ser/Thr where X can be any amino acid except proline and aspartic acid [44]. All N-glycans share a common minimal structure  $\text{Man}_3\text{GlcNAc}_2$  constituted of a N, N'-diacetyl chitobiose unit, a  $\beta$ -mannose residue linked to the chitobiose and two  $\alpha$ -mannose residues linked to hydroxyl 3 and 6 of the  $\beta$ -mannose [44]. According to the substitutions of this core, plant N-glycans have so far been classified into two categories: the high-mannose-type and complex-type N-glycans. The recent analysis of plant complex N-glycan structures has revealed a diversity which has been ignored for a long time. New structures have been recently described with the introduction of new technologies and the use of powerful equipment, such as nuclear magnetic resonance and mass spectrometry in a field where most results up to the late 1980s were obtained from enzymatic sequencing coupled to low-performance liquid chromatography. From recent results on plant N-linked oligosaccharides, we propose the redefinition of the plant N-glycan classification into the four following classes: high-mannose-type, complex-type, paucimannosidic-type and hybrid-type N-glycans.

#### High-mannose-type N-glycans

High-mannose-type N-glycans from  $\text{Man}_5\text{GlcNAc}_2$  to  $\text{Man}_9\text{GlcNAc}_2$  arise from the limited trimming of Glc and Man residues from the precursor oligosaccharide  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  (Figure 1a). High-mannose-type N-glycans were first identified in plants in soybean agglutinin [49]. They were then found N-linked to various extracellular and vacuolar glycoproteins. High-mannose-type N-glycans have also recently been identified as the unique N-linked oligosaccharides of spinach [52] or maize calreticulin (P. Lerouge *et al.*, unpublished results), a glycoprotein-specific chaperone that resides in the plant endoplasmic reticulum (ER).

#### Complex-type N-glycans

As in other eukaryotic cells, plant complex-type N-glycans result from the processing in the Golgi apparatus of high-mannose-type N-glycans by action of specific glycosidases and glycosyltransferases. Complex-type plant N-glycans are characterized by the presence of  $\alpha(1,3)$ -fucose and/or a  $\beta(1,2)$ -xylose residues respectively linked to the proximal N-acetyl glucosamine and to the  $\beta$ -mannose residues of the core and by the presence of  $\beta(1,2)$ -N-acetyl glucosamine residues linked to the  $\alpha$ -mannose units (Figure 1b). Recently, larger complex-type plant N-glycans were

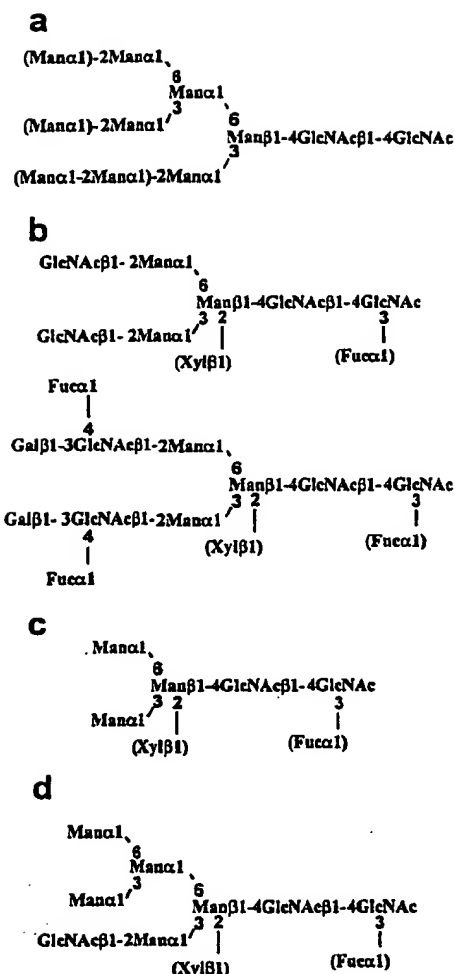


Figure 1. Structures of (a) high-mannose-type N-glycans  $\text{Man}_5\text{GlcNAc}_2$  to  $\text{Man}_9\text{GlcNAc}_2$ , (b) complex-type N-glycans, (c) paucimannosidic-type N-glycans  $\text{Man}_3(\text{Xyl})(\text{Fuc})\text{GlcNAc}_2$  and (d) hybrid-type N-glycans  $\text{GlcNAcMan}_5(\text{Xyl})(\text{Fuc})\text{GlcNAc}_2$  isolated from plant glycoproteins.

identified containing additional  $\alpha(1,4)$ -fucose and  $\beta(1,3)$ -galactose residues linked to the terminal N-acetyl glucosamine units [26, 51]. These modifications yield  $\text{Gal}\beta 1-3(\text{Fuc}\alpha 1-4)\text{GlcNAc}$  sequences known as Lewis a ( $\text{Le}^a$ ) antigens and usually found on cell-surface glycoconjugates in mammals (Figure 1b). Such structures have been previously isolated from sycamore laccase [81], miraculin [82] and from a pollen allergen isolated from *Cryptomeria japonica* [54]. However, the structures reported in these paper

were incorrect and re-examined in recent papers as mentioned above [26, 51].

#### *Paucimannosidic-type N-glycans*

This nomenclature was previously proposed for modified insect N-linked glycans deprived of terminal N-acetyl glucosamine residues linked to the  $\alpha$ -mannose residues of the core [2]. By comparison, we propose to give the name paucimannosidic-type N-glycans to modified plant oligosaccharides having only an  $\alpha(1,3)$ -fucose and/or a  $\beta(1,2)$ -xylose residue linked, respectively, to the proximal GlcNAc and the  $\beta$ -mannose residues of the core  $\text{Man}_3\text{GlcNAc}_2$  (Figure 1c) or to the restricted core  $\text{Man}_2\text{GlcNAc}_2$ . These glycans, previously described as PHA-type N-glycans, have been identified not only in bean phytohemagglutinin (PHA) but also in various other plant glycoproteins [3, 4, 11, 14, 16, 28, 31, 32, 36, 42, 43, 45, 55, 57, 58, 75, 77, 93, 97]. Paucimannosidic-type N-glycans result from the elimination of terminal residues from complex-type N-glycans and can be considered as typical vacuole-type N-glycans as developed later in this review.

#### *Hybrid-type N-glycans*

Hybrid-type N-glycans result from the processing of only the  $\alpha(1,3)$ -mannose branch of the intermediate  $\text{Man}_5\text{GlcNAc}_2$  leading to oligosaccharides having  $\alpha(1,3)$ -fucose and/or a  $\beta(1,2)$ -xylose residues linked to  $\text{GlcNAcMan}_5\text{GlcNAc}_2$  (Figure 1d) [58].

#### **What has been (really) shown on N-glycan biosynthesis and maturation in plants?**

The N-glycosylation of plant proteins starts in the ER with the transfer by the oligosaccharyl transferase of the oligosaccharide precursor  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  from a dolichol lipid carrier to specific Asn residues on the nascent polypeptide chain. The precursor is subsequently modified by glycosidases and glycosyltransferases during the transport of the glycoprotein downstream in the secretory pathway to its final localization. As illustrated in Figure 2, the oligosaccharide precursor  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  first undergoes an early trimming of the three terminal glucose units catalysed by the glucosidases I and II in the ER [40, 78]. A transient reglucosylation by an ER UDP-glucose:glycoprotein glucosyltransferase may occur subsequently to the elimination of these three glucose residues [59, 85]. This reglucosylation has been

shown to act on unfolded proteins and to be involved in the quality control of glycoproteins in the ER [33, reviewed by Galili *et al.*, this issue]. In mammals, prior to the trimming of mannose residues by the Golgi  $\alpha$ -mannosidase I, an ER mannosidase specifically removes a single mannose residue to yield  $\text{Man}_8\text{GlcNAc}_2$ . Such an ER mannosidase has not been detected in plants so far. However, the structures of the major N-linked glycans of an ER resident chaperone, calreticulin, purified either from the spinach [52] or from maize (P. Lerouge *et al.*, unpublished results), were identified as  $\text{Man}_8\text{GlcNAc}_2$  and  $\text{Man}_{9-8}\text{GlcNAc}_2$ , respectively, which indicates that a specific mannosidase could also be involved in the processing of plant N-linked glycans within the ER.

As illustrated in Figure 2, plant N-glycans can be further modified in the Golgi into complex-type N-glycans during the transport of the glycoprotein from the *cis*, through medial to *trans* cisternae. First, the  $\alpha$ -mannosidase I ( $\alpha$ -Man I) removes one to four  $\alpha(1,2)$ -mannose residues and converts  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_5\text{GlcNAc}_2$  [74, 79]. Then, the biosynthesis of complex-type N-glycans starts with the addition of a first N-acetylglucosamine residue to the  $\alpha(1,3)$ -mannose branch of the  $\text{Man}_5\text{GlcNAc}_2$  high-mannose-type glycan. This step is catalysed by the N-acetylglucosaminyltransferase I (GNT I) to yield  $\text{GlcNAcMan}_5\text{GlcNAc}_2$  [39, 83]. Two additional mannose residues are then removed from  $\text{GlcNAcMan}_5\text{GlcNAc}_2$  by the  $\alpha$ -mannosidase II ( $\alpha$ -Man II) [41] and another outer N-acetylglucosamine residue is transferred by the N-acetylglucosaminyltransferase II (GNT II) to the  $\alpha(1,6)$ -mannose branch [39, 83]. At this stage,  $\alpha(1,3)$ -fucosylation and  $\beta(1,2)$ -xylosylation of the core  $\text{Man}_3\text{GlcNAc}_2$  may occur to yield plant specific N-linked glycans. The study of the substrate specificity of the  $\alpha(1,3)$ -fucosyltransferase ( $\alpha(1,3)$ -FucT) and the  $\beta(1,2)$ -xylosyltransferase ( $\beta(1,2)$ -XylT) has shown that the presence of at least one terminal N-acetylglucosamine residue is a prerequisite for the transfer of  $\alpha(1,3)$ -fucose and  $\beta(1,2)$ -xylose [39, 72, 83, 101]. The  $\alpha(1,3)$ -FucT [72] and the  $\beta(1,2)$ -XylT [101] have been purified from the microsomal fraction of mung bean seedlings but these enzymes have not been cloned yet. The sequences of the xylosylation and the fucosylation events are not completely understood. Plant N-linked glycans having only a  $\beta(1,2)$ -xylose or only an  $\alpha(1,3)$ -fucose residue have been identified in plant glycoproteins [14, 16, 34, 55, 75]. Moreover, the substrate specificities of the  $\alpha(1,3)$ -FucT and  $\beta(1,2)$ -XylT are

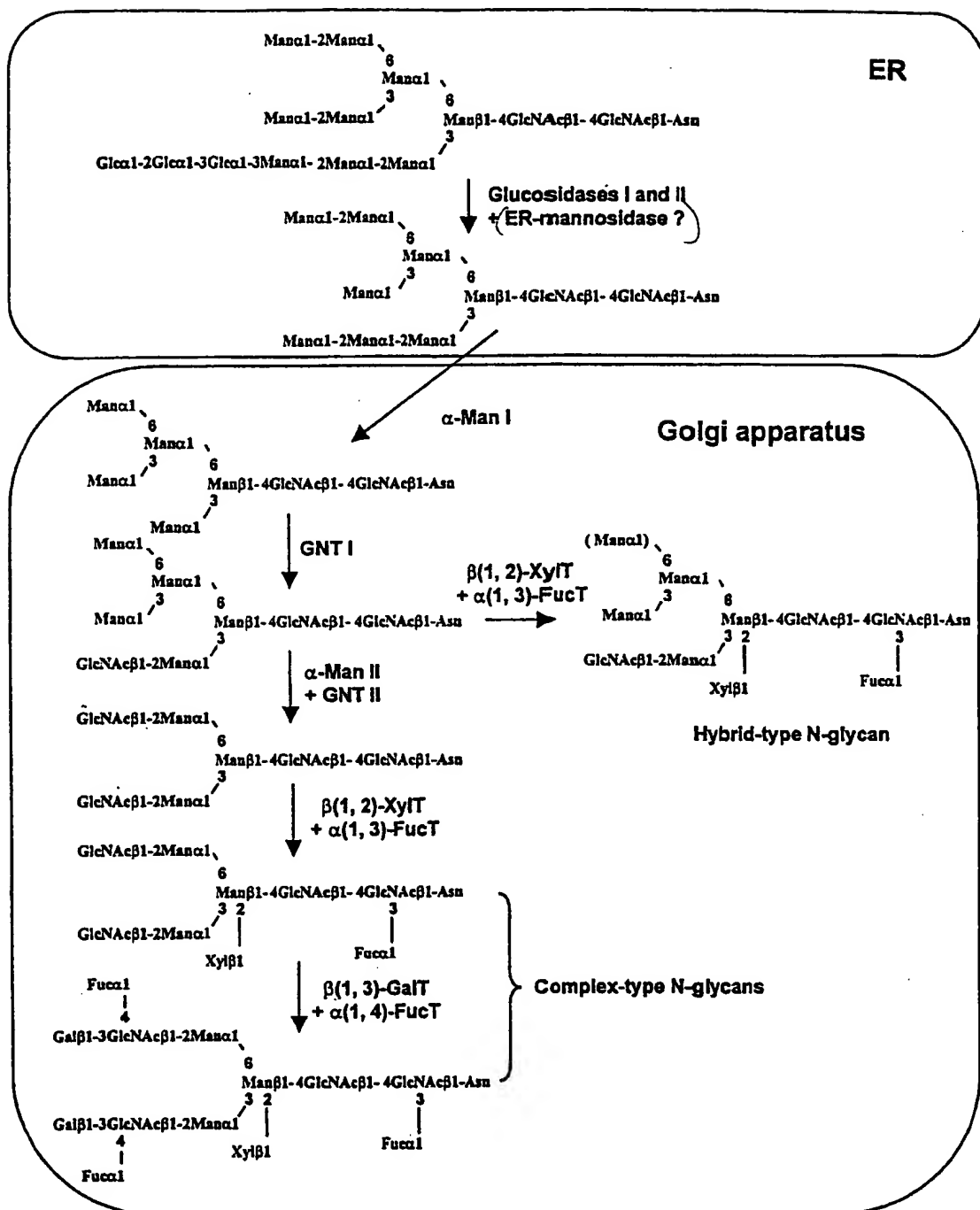


Figure 2. Processing of plant N-linked glycans in the endoplasmic reticulum (ER) and the Golgi apparatus. Abbreviations:  $\alpha$ -Man I,  $\alpha$ -mannosidase I;  $\alpha$ -Man II,  $\alpha$ -mannosidase II; GNT I, N-acetylglucosaminyltransferase I; GNT II, N-acetylglucosaminyltransferase II;  $\beta$ (1,2)-XylT,  $\beta$ (1,2)-xylosyltransferase;  $\alpha$ (1,3)-FucT,  $\alpha$ (1,3)-fucosyltransferase;  $\beta$ (1,3)-GalT,  $\beta$ (1,3)-galactosyltransferase;  $\alpha$ (1,4)-FucT,  $\alpha$ (1,4)-fucosyltransferase.

not affected, respectively, by the absence of the  $\beta(1,2)$ -xylose or  $\alpha(1,3)$ -fucose residues linked to the core [39, 72, 83, 101]. Consequently,  $\alpha(1,3)$ -fucosylation and  $\beta(1,2)$ -xylosylation appear to be independent events. Using an immunocytochemical approach to study the subcompartmentation of the  $\beta(1,2)$ -xylosylation and  $\alpha(1,3)$ -fucosylation events, we have demonstrated that these two steps occur mostly in the medial and in the *trans* Golgi cisternae, respectively. This indicates that during the transport of the glycoprotein through the Golgi apparatus, the  $\beta(1,2)$ -xylose transfer starts before the addition of the  $\alpha(1,3)$ -fucose residue to the core [25]. *In vitro*,  $\alpha(1,3)$ -FucT and  $\beta(1,2)$ -XylT could also act on GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> substrate leading to plant hybrid-type N-glycans. As a consequence, hybrid structures *in planta* could result from an uncompleted action of  $\alpha$ -Man II. This was recently demonstrated in our laboratory by studying the effect of swainsonine, an inhibitor of  $\alpha$ -Man II. After swainsonine treatment, only hybrid oligosaccharides were identified (P. Lerouge *et al.*, unpublished results).

After the transfers of xylose and fucose on the core, complex-type N-glycans can be further processed by addition of terminal fucose and galactose residues to yield one or two antennae constituted of Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc sequences as shown in Figure 1b [26, 51]. This sequence, known as Lewis a ( $Le^a$ ) antigen, is usually found on cell-surface glycoconjugates in mammals and is involved in cell-cell recognition and cell adhesion processes. These new plant antigens result from the addition of fucose and galactose residues by a  $\beta(1,3)$ -galactosyltransferase ( $\beta(1,3)$ -GalT) and an  $\alpha(1,4)$ -fucosyltransferase ( $\alpha(1,4)$ -FucT) on terminal N-acetylglucosamine residues of complex-type N-glycans. The study of the substrate specificity of the  $\alpha(1,4)$ -FucT has shown that this enzyme specifically transfers fucose from GDP-fucose to Gal $\beta$ 1-3GlcNAc [15, 26, 51].

After maturation in the ER and the Golgi apparatus, complex-type N-glycans can be further modified during the glycoprotein transport to, or in, the compartment of its final destination. For example, the terminal glucosamine residues attached to the complex-type N-glycan of phytohemagglutinin and phaseolin were found to be removed during the transport to the vacuole, or within this lytic compartment, by action of a N-acetylglucosaminidase as shown in Figure 3 [75, 89]. In addition, most vacuolar glycoproteins and seed storage glycoproteins described so far, were found to be N-glycosylated with the

same kind of modified N-glycans containing fucose and/or xylose residues but devoid of terminal glucosamine residues. As mentioned earlier, we propose to name paucimannosidic-type N-glycans these typical vacuole-type oligosaccharide side (Figure 1c). Because we know that the presence of terminal glucosamine residues is a prerequisite for the transfer of the  $\alpha(1,3)$ -fucose and the  $\beta(1,2)$ -xylose residues, paucimannosidic-type N-glycans can only result from post-Golgi modifications occurring on complex-type N-glycans. From a similar process, modified hybrid Man<sub>4</sub>Xyl(Fuc)GlcNAc<sub>2</sub> and Man<sub>5</sub>Xyl(Fuc)GlcNAc<sub>2</sub> [31, 43, 97] could also result from degradation in the vacuole of the intermediate hybrid-type N-glycan described in Figure 2. In contrast, extracellular glycoproteins, such as sycamore laccase [26, 81], miraculin [82], the pollen allergen *Cry j I* [54] and an extracellular peroxidase from *Vaccinium myrtillus* [51] were found to be N-glycosylated mostly by complex-type N-glycans including  $Le^a$ -containing oligosaccharides. Paucimannosidic-type N-glycans linked to vacuolar glycoproteins could also result from the degradation of larger  $Le^a$ -containing N-glycans, by successive action of exoglycosidases in a prevacuolar compartment or in the vacuole as proposed in Figure 3. We are currently investigating whether vacuolar glycoproteins transiently acquire  $Le^a$  structures before further trimming during their transport to or within the vacuole. This will help us to determine whether the  $Le^a$  biosynthesis is a common feature of plant glycoproteins or whether it occurs exclusively on extracellular glycoproteins suggesting that some N-glycan processing events could be specific to the final destination of the protein. The complex oligosaccharides may also be partially degraded by exoglycosidases in the extracellular compartment as demonstrated in Tezuka *et al.* [84], but the rate of degradation appears to be lower than observed in the vacuole.

### Heterogeneity of the N-glycosylation

As discussed above, oligosaccharyl transferase, glycosyltransferases and glycosidases successively transfer and modify N-linked glycans in the ER and during the transport of the glycoprotein to its final destination. However, the structural analysis of glycoproteins has revealed the large heterogeneity of the N-glycosylation in plants indicating that various parameters could affect the efficiency of the N-glycosylation machinery. This heterogeneity is observed at three

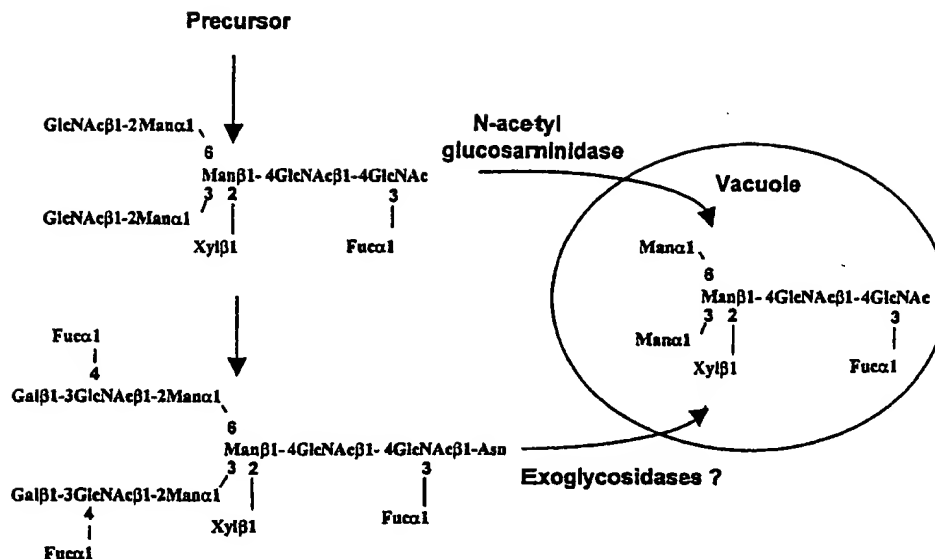


Figure 3. Modifications of complex-type N-glycans in the plant vacuole.

different levels: the number of glycan side-chains, the extent of glycan modification of the different side chains of the same glycoprotein and the heterogeneity of oligosaccharide structures on the same N-glycosylation site. In the early steps of the N-glycosylation, an uncompleted transfer in the ER of the oligosaccharide precursor by the oligosaccharyl transferase can occur, leading to the biosynthesis of glycoproteins having various numbers of N-glycans. For instance, one of the two glycosylation sites of the bean storage protein phaseolin is partially used during this glycoprotein biosynthesis. As a consequence, mature phaseolin bears either one or two oligosaccharide side chains [7]. Factors that can affect the efficiency of use of N-glycosylation sites are reviewed by Galili *et al.* (this issue). The structural analysis of mature glycoproteins has shown that some glycoproteins have exclusively glycans of the high-mannose-type or have both high-mannose-type and complex- or paucimannosidic-type N-glycans on different N-glycosylation sites. This means that on a same glycoprotein, some oligosaccharide side-chains can be processed in the Golgi apparatus and in post Golgi compartments while others cannot. The extent of glycan modifications is related to their physical accessibility to the processing enzymes. For example, PHA, the bean lectin, is mostly N-glycosylated by one paucimannosidic-type oligosaccharide on Asn-60 and by high-mannose-type oligosaccharides on Asn-

60 [73, 77, 89]. This latter side-chain has low accessibility to processing enzymes and remains unmodified on mature PHA while the oligosaccharide N-linked to Asn-60 is highly accessible to processing enzymes. This illustrates that protein conformation is a major determinant in the extent of oligosaccharide modifications [18]. The importance of glycan accessibility in the extent of maturation by processing enzymes has also been clearly demonstrated through the analysis of bean phaseolin glycosylation. As mentioned above, phaseolin consists in homologous polypeptides having either one or two glycans. The diglycosylated phaseolin has high-mannose-type N-glycans attached to both Asn-252 and Asn-341 whereas monoglycosylated phaseolin has a paucimannosidic-type N-glycan attached to Asn-252. This implies that the extent of glycan processing on Asn-252 is controlled by Asn-341 glycosylation status, suggesting that the absence of glycan on Asn-341 may result in a greater accessibility to the processing enzymes of the oligosaccharide attached to Asn-252 [75]. In addition to the heterogeneity resulting from the number and the extent of processing of oligosaccharide side-chains on a glycoprotein, recent detailed analysis of glycan distribution on the different glycosylation sites of a glycoprotein has revealed that even on a same site of a glycoprotein the glycan structures can be heterogeneous [14, 51, 57, 64, 76, 82]. This heterogeneity results from both the partial modification of the glycans

by Golgi processing enzymes and glycan degradation by exoglycosidases in the compartment where the glycoprotein accumulates.

#### **N-glycans: milestones along the plant secretory pathway**

When the N-glycans attached to a plant protein are fully accessible to processing enzymes, these oligosaccharide side-chains are matured into paucimannosidic- or complex-type N-glycans during the transport of the glycoprotein. These oligosaccharide structures, resulting from successive N-glycan processing in ER, in the Golgi apparatus and in post-Golgi compartments, can serve as useful markers of glycoprotein transport through the plant secretory pathway, as is illustrated in Figure 4. For instance, most vacuolar glycoproteins and seed storage glycoproteins described so far were found to be N-glycosylated with paucimannosidic-type N-glycans containing fucose and/or xylose residues but devoid of terminal glucosamine residues, whereas most extracellular glycoproteins bear complex-type N-glycans with terminal N-acetyl glucosamine residues or Le<sup>a</sup> antennae (Figure 4). This indicates, as demonstrated for bean storage proteins [75, 89], that post-Golgi trimming of terminal glucosamine units on complex-type N-glycans could be considered as a common feature in the maturation of vacuolar glycoproteins. The use of N-glycans as milestones along the secretory pathway is also illustrated through the recent description of the N-glycosylation of ER-resident glycoproteins. In mammals and yeast, the structures of oligosaccharides N-linked to ER-resident glycoproteins result from both ER and Golgi maturations arising from the continual retrieval of these glycoproteins from an early or late Golgi compartment back to the ER. For instance, as illustrated in Figure 5, bovine brain or rat liver calreticulins exhibit N-glycan modifications such as extensive trimming or addition of terminal galactose residues that are known to specifically occur in the early Golgi apparatus and even further downstream in the *trans* Golgi [53]. In contrast, the structures of the major N-linked glycans of a plant ER resident chaperone, calreticulin, purified either from the spinach [52] or from maize (Lerouge *et al.*, unpublished results), have been identified as Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>9-8</sub>GlcNAc<sub>2</sub>, respectively (Figure 5). The lack of complex N-linked glycans attached to these plant calreticulins does not definitively exclude their recycling

from post-ER compartments back to the ER. However, these data strongly suggest that either the recycling efficiency is so high in plants that these calreticulins are not travelling very far downstream before their transport back to the ER, or that this travel only occurs for a minority of them. Another explanation could be that plant calreticulins studied so far have oligosaccharide side-chains that are not accessible to Golgi processing enzymes and remain unmodified during recycling. This latter hypothesis is currently being studied with ER-resident reporter glycoproteins previously shown to bear highly accessible N-glycans.

#### **Last update on plant and organ-specific N-glycosylation: thanks to a reporter (glycoprotein)**

The production of reporter glycoproteins in different plant expression systems offers an opportunity to compare plant and organ specific maturation of N-glycans. This powerful approach was recently illustrated using the bean phytohaemagglutinin (PHA) as a reporter glycoprotein [64, 65]. In this study, the well known plant glycoprotein PHA, a lectin that accumulates in bean cotyledon, was expressed in different transgenic plant systems. It had previously been shown that PHA is mostly N-glycosylated by two oligosaccharide side-chains: a high-mannose-type N-glycan Man<sub>8</sub>GlcNAc<sub>2</sub> attached to Asn-12 and a paucimannosidic-type N-glycan Man<sub>3</sub>XylFucGlcNAc<sub>2</sub> (see Figure 1c) attached to Asn-60 [73, 77, 89]. Further studies have shown that these glycans have different maturations because of their different accessibility to Golgi processing enzymes [18]. A recent study has illustrated that glycan heterogeneity on bean PHA is not only side-chain-dependent, but that high-mannose glycans are also very heterogeneous with structures from Man<sub>6</sub>GlcNAc<sub>2</sub> to Man<sub>9</sub>GlcNAc<sub>2</sub> (Man-6 to Man-9) [64]. Furthermore, the analysis of the carbohydrate profiles of recombinant PHA, produced in tobacco suspension-cultured cells or tobacco and *Arabidopsis* plants, has shown that the heterogeneity of high-mannose-type N-glycans linked to Asn-12, as well as the structure of the paucimannosidic-type N-glycan Man<sub>3</sub>XylFucGlcNAc<sub>2</sub> linked to Asn-60, are conserved in all plant expression systems used in this study [65] (Figure 6). However, some differences such as the ratio between high-mannose glycans Man<sub>6</sub>GlcNAc<sub>2</sub> and Man<sub>9</sub>GlcNAc<sub>2</sub> (Man-6 to Man-9) and the presence of a minor non-fucosylated

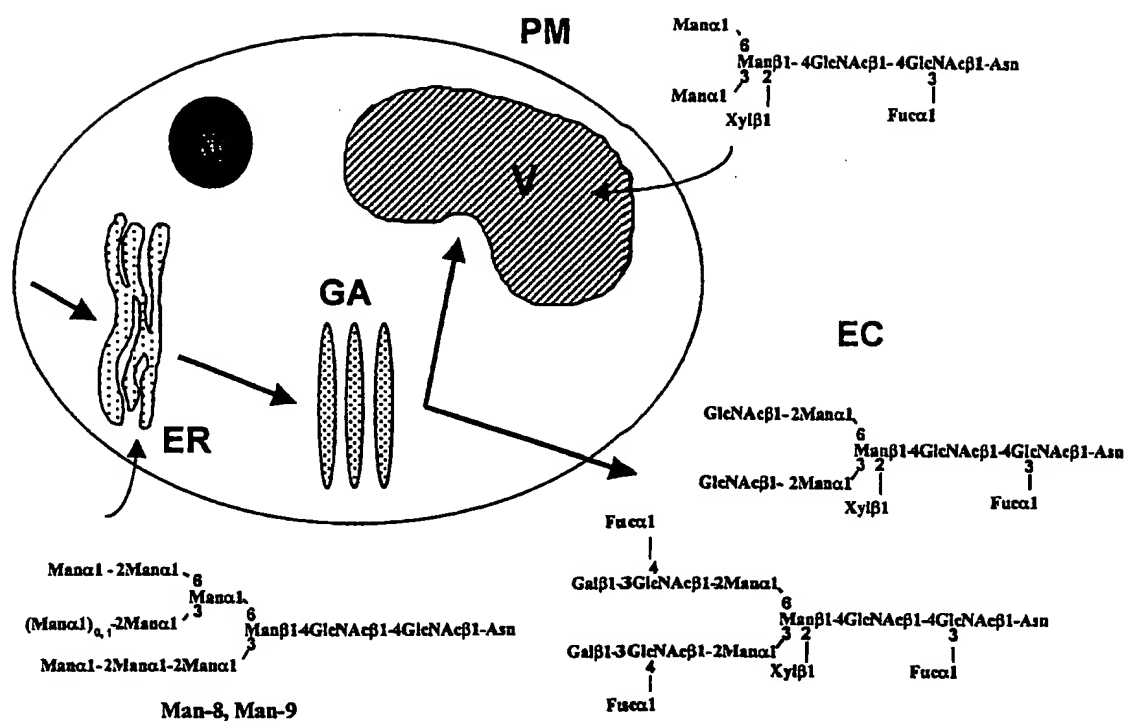


Figure 4. Distribution of N-glycan structures in the plant cell. Abbreviations: EC; extracellular compartment (including cell wall), ER; endoplasmic reticulum, GA; Golgi apparatus, N; nucleus, PM; plasma membrane, V; vacuole.

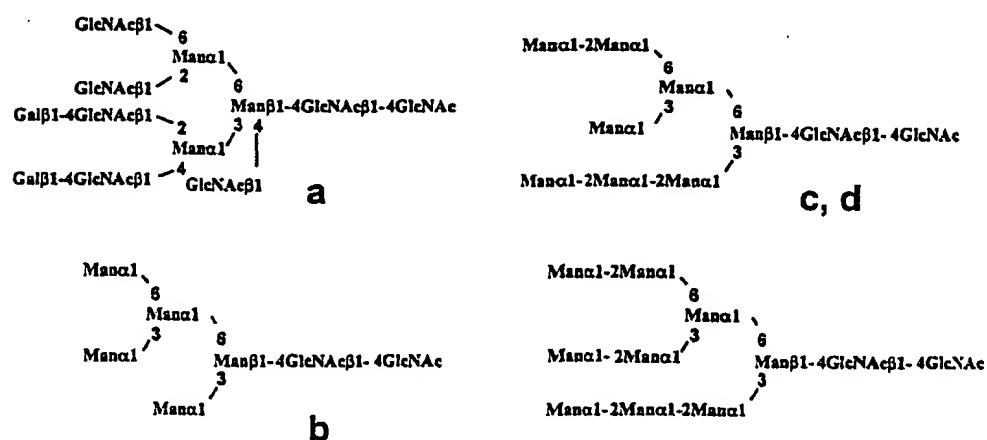


Figure 5. Structures of oligosaccharides N-linked to calreticulins from mammals or plants. Rat liver (a) and bovine brain calreticulins (b), spinach (c) and maize (d) calreticulins.



glycan linked to Asn-60, were observed, suggesting that the levels of Golgi mannosidase and fucosyltransferase activities differ in the plant heterologous expression systems used (Figure 6). Using a similar approach, the N-glycosylation of recombinant PHA, isolated from the different organs of transgenic tobacco plants, was analysed to investigate whether or not the N-glycosylation of a vacuolar glycoprotein is organ-specific in plants [65]. This study has shown that the structures of PHA glycans are similar when matured in flower, fruit, limb, petiole, stem or root of transgenic tobacco plants. As a consequence, when using PHA as a reporter glycoprotein, it appears that the maturation of N-linked glycans attached to this model vacuolar glycoprotein is highly conserved in plants and in the different organs of a same plant.

#### Is *Arabidopsis thaliana* another useful weed for plant glycobiology?

Mutants have been widely used for biochemical studies of the N-glycosylation in yeast and mammals. Although a rapidly increasing number of mutants are available in several plant species, and particularly in *Arabidopsis thaliana*, only two of them present a clearly identified mutation affecting the biosynthesis of N-linked glycans. The first one, the *A. thaliana cgl* mutant which lacks the N-acetylglucosaminyltransferase I (GNT I) activity, is unable to synthesize complex-type N-glycans and accumulates  $\text{Man}_5\text{GlcNAc}_2$  oligosaccharides as illustrated in Figure 7. This study has confirmed that GNT I, as in other eukaryotes, is the key enzyme in the biosynthesis of complex-type N-glycans [91]. Another *A. thaliana* mutant, the *mur1* mutant, does not synthesise L-fucose [67]. This mutant was found to be affected in the gene encoding for a GDP-D-mannose-4,6-dehydratase, an enzyme involved in the biosynthesis of L-fucose [8]. The structure of cell wall polysaccharides from *Arabidopsis* plants carrying the *mur1* mutation has been investigated recently [99] and compared to cell wall polysaccharides from wild-type plants [98]. This study has shown that, in the xyloglucan of this fucose-deficient mutant, L-fucose is replaced by L-galactose, a monosaccharide structurally similar to L-fucose, without affecting the biological activity of fucose-containing oligosaccharides derived from these polymers. We have analysed the structures of N-linked oligosaccharides in the *mur1* mutant and found that L-fucose is partially re-

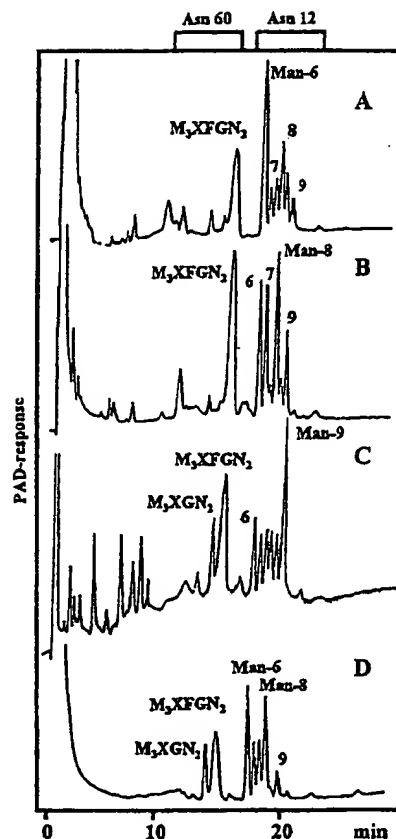


Figure 6. High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) profiles of N-glycans isolated from bean PHA (A) and from PHA expressed in suspension-cultured tobacco cells (B), tobacco plants (C) and *Arabidopsis thaliana* plants (D). Abbreviations: Asn 60 and Asn 12; glycans N-linked to Asn-60 and Asn-12. Man-6 or 6;  $\text{Man}_6\text{GlcNAc}_2$ , Man-7 or 7;  $\text{Man}_7\text{GlcNAc}_2$ , Man-8 or 8;  $\text{Man}_8\text{GlcNAc}_2$ , Man-9 or 9;  $\text{Man}_9\text{GlcNAc}_2$ ,  $\text{M}_3\text{XGN}_2$ ;  $\text{Man}_3\text{XylGlcNAc}_2$ ,  $\text{M}_3\text{XFGN}_2$ ;  $\text{Man}_3\text{XylFucGlcNAc}_2$ .

placed by L-galactose as observed in the cell-wall xyloglucan [66]. This demonstrates that, in absence of L-fucose, the  $\alpha(1,3)$ -fucosyltransferase is able to transfer L-galactose from GDP-L-galactose, instead of fucose from GDP-L-fucose, to the proximal N-acetyl glucosamine residue of the core, leading to plant N-glycans having a new core  $\text{Man}_3\text{Xyl}(\text{L-Gal})\text{GlcNAc}_2$  as represented in Figure 7.

A larger scale screening for new mutants is now facilitated by the detailed characterization of new glycan-specific antibody probes. These probes were developed taking advantage of the high immunogenicity of plant complex N-glycans. Some of these probes

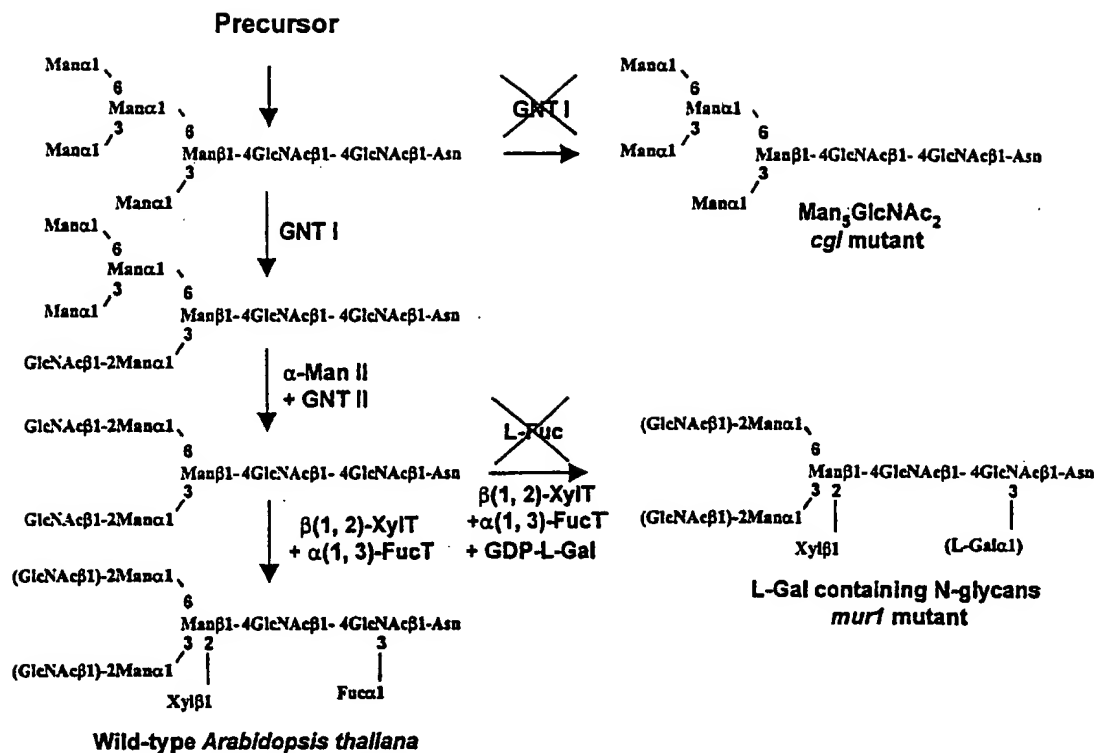


Figure 7. Processing of the N-linked glycans in wild-type *Arabidopsis thaliana*, and in the *cgl* and the *murI* mutants. Abbreviations: see Figure 2.

are commercially available as crude antibody preparation obtained from rabbits immunised with plant or insect glycoproteins and can be easily affinity-purified as described in Faye *et al.* [22] and Fitchette-Lainé *et al.* [27]. Antibodies specific for carbohydrate epitopes of plant modified glycans, i.e.  $\alpha(1,3)$ -fucose,  $\beta(1,2)$ -xylose, have been characterized in detail [22]. Recently, antibodies to plant Le<sup>a</sup>-containing N-glycans have been identified [26]. The use of these plant glycan-specific antibodies, particularly for the screening of tagged mutants of *A. thaliana*, will not only provide further information on the biosynthesis of complex glycans in plants, but they will also help in the cloning of glycosyltransferases involved in the maturation of these structures. Up to now, no plant glycosyltransferase sequence is available in the data banks. In addition, no antibodies specific for Golgi transferases or for any other protein specific for the plant Golgi are available. In this respect, the plant glycan-specific antibodies are so far the only probes

available to immunolocalize the Golgi apparatus in the plant cell [25, 35].

Besides the advantage of *A. thaliana* as a reservoir of potentially interesting mutants, this plant provides only very limited amount of material for structural analysis of glycoprotein N-linked glycans due to the small size of the mature plants. As a consequence, up to now *A. thaliana* is not very well adapted for glycan analysis although this limitation could be rapidly overcome in a field where the rapid progress of technologies now allows detailed structural analysis from less than 10  $\mu$ g of purified oligosaccharides. The strongest limitation in the use of *A. thaliana* as a model system in plant glycobiology is related to its very little diversity in complex glycan structures which probably reflects the minimum equipment of this plant in Golgi glycosyltransferases. For example, a comparison of complex glycan structures identified in *A. thaliana* and sycamore cells suggests that *A. thaliana* is unable to build detectable amounts of complex-type Le<sup>a</sup>-containing N-glycans [26, 66]. This plant seems

to be limited in the biosynthesis of glycans of limited size, having only terminal glucosamine residues attached the  $\alpha$ -mannose branches, as illustrated in Figure 7 [66]. This apparent inability in biosynthesis of large oligosaccharides has not been observed in other plants [26].

#### N-glycan functions in plants: a little more than umbrellas

In eukaryotes, N-linked glycans have numerous roles. Some of them, such as a prevention of proteolytic degradation or induction of the correct folding of the protein, often directly depend on the large dimension of oligosaccharides covering the protein backbone in an umbrella-like manner. N-linked oligosaccharides may also contain targeting information, or may be directly involved in protein recognition or cell-cell adhesion processes. In plants, the roles of N-glycans have been studied using different approaches, such as the use of N-glycosylation and N-glycan-processing inhibitors, site-directed mutagenesis of N-glycosylation sites or the study of mutants affected in the maturation of N-glycans. In these three strategies, either the N-glycosylation is completely suppressed or only the processing to complex-type N-glycans is inhibited. In both cases, the analysis of the effects of these alterations have provided some information concerning the roles of the N-glycosylation and of the N-glycan processing in plants.

In plants, N-linked glycans strongly influence the glycoprotein conformation, stability and biological activity. In the early steps of the protein biosynthesis, when the nascent polypeptide enters the lumen of the ER and the oligosaccharyl transferase attaches the oligosaccharide precursor to specific asparagine residues, the presence of N-glycans strongly affects both the co- and post-translational folding of the protein. Numerous works have also demonstrated that N-glycans can protect the protein from proteolytic degradations, as well as that they are responsible for the thermal stability, solubility and biological activity of glycoproteins. For example, concanavalin A (Con A), the lectin from jack bean seeds, is synthesised as a glycosylated inactive prolectin, the pro-ConA. *In planta*, the processing of pro-Con A into Con A occurs in protein bodies and involves two endoproteolytic events to excise a glycopeptide from the centre of the pro-Con A molecule followed by the ligation of the polypeptide chains [9, 12]. Inhibition of the

pro-Con A N-glycosylation strongly reduces the prolectin solubility and slows down its transport from ER to the protein storage vacuole [19]. Furthermore, pro-Con A, which has no lectin activity, could be converted into a carbohydrate-binding protein after *in vitro* deglycosylation with peptide N-glycosidase [68]. Both results indicate that the N-glycosylation of Con A is important for its transport to the vacuole and the regulation of its lectin activity. The regulation of the lectin activity is crucial since a lectin with high affinity for high-mannose-type N-glycans, such as the mature Con A, will probably bind to newly synthesized N-linked glycoproteins in the ER preventing their transport downstream in the secretory pathway.

In animal cells, oligosaccharide side chains may act as a targeting signal for lysosomal hydrolases [69]. The high-mannose-type N-glycans of these glycoproteins are phosphorylated in the *cis*-Golgi compartment and the resulting mannose-6-phosphate-containing glycoprotein is recognised by membrane receptors and subsequently transported to lysosomes. The function of N-glycans in the targeting of proteins to the vacuole, the plant equivalent of animal lysosome, has been extensively studied. Plant protein N-glycans are not phosphorylated in the Golgi apparatus and mannose-6-phosphate receptors have not been identified in plants [30]. Furthermore, with a few exceptions, as for pro-ConA, the targeting of vacuolar glycoproteins has been found to be unmodified in presence of N-glycosylation inhibitors [6]. The expression in transgenic tobacco plants of a PHA cDNA mutagenised on both N-glycosylation sites did not alter the accumulation of the unglycosylated lectin in the protein bodies of transgenic tobacco seeds [90]. The intracellular transport of several other vacuolar glycoproteins have been investigated using similar approaches. All results have led to the conclusion that N-linked glycans have no specific role in the targeting of plant glycoproteins to the vacuole.

The function of oligosaccharide side-chains in the secretion of plant glycoproteins into the extracellular compartment has also been investigated. In presence of tunicamycin, a N-glycosylation inhibitor, most extracellular N-glycosylated glycoproteins are not secreted any more [17, 21]. This result is consistent with the observation that the unglycosylated carrot cell-wall  $\beta$ -fructosidase is degraded during its transport in the secretory pathway or immediately after its arrival in the wall [21]. However, it has been shown that the processing of high-mannose-type to complex-type N-glycans is not required for transport and secre-

tion of extracellular glycoproteins in plants [17, 48]. For instance, in the presence of the glycan processing inhibitor, castanospermine, N-glycosylation with immature  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  oligosaccharide side-chains is sufficient for extracellular glycoproteins from sycamore cells to be successfully transported through the quality control system, and finally secreted [48]. In contrast with the secretion defect of these glycoproteins in the presence of glycosylation inhibitors, this result clearly shows that while glycosylation is absolutely required for secretion of extracellular glycoproteins by sycamore cells, the structure of their oligosaccharides is not the basis of the quality control system which acts when the N-glycosylation of proteins is prevented. Beyond this function which could be related to the role of N-glycosylation in protein folding in the ER, our results are also consistent with the hypothesis proposed by Fiedler and Simons [24] that glycans could target glycoproteins to the cell surface. In this respect, as proposed for the secretory protein sorting in animal cells, the internal core residues instead of the peripheral epitopes of N-glycans could act as sorting signals for plant extracellular glycoproteins. New strategies are currently developed to investigate whether, when the N-glycosylation is inhibited, the prevention of glycoprotein secretion is correlated to a misfolding of the protein, to the absence of a core glycan targeting signal or to the degradation of the protein by proteases during the intracellular transport or after the secretion in the extracellular compartment.

It was recently shown in mammalian and yeast cells that misfolded, unassembled proteins or unglycosylated glycoproteins produced in the presence of tunicamycin are rapidly degraded by the proteasomes after their retrograde transport from the ER lumen back into the cytosol [37, 94, 96]. Such a process could also occur for misfolded plant extracellular glycoproteins when they are synthesised without their N-glycans. This would implicate that some structural features on these proteins functioning as degradation motifs are not present on vacuolar glycoproteins previously studied.

After their retrograde transport to the cytosol and before their degradation by proteasomes, misfolded mammalian glycoproteins are deglycosylated by a cytosolic N-glycanase [96]. This deglycosylation probably generates most free high-mannose-type oligosaccharides already described in the cytosol of mammalian cells. Free N-glycans have also been identified in plants. They were originally found in the culture medium of suspension-cultured cells of *Silene alba*

[60]. The origin of these free glycans and the sub-cellular localisation of their release have not been clearly elucidated in plants. According to their structures [63], plant unconjugated N-glycans could result from degradation either of glycolipid intermediates or of glycoproteins in the cytosol, a pre- or a post-Golgi compartment. There are several reports illustrating the biological activity of these free glycans in plant metabolism. For example, they were shown to stimulate ripening of tomato fruits [62] and to act as growth factors during the development of flax seedlings [61].

With the exception of free glycans, all N-glycan functions identified so far in plants depend on their presence on a protein, independently of their oligosaccharide structure. This is more generally illustrated from studies on the viability of glycosylation mutants or of plants grown on glycosylation inhibitors. The use of tunicamycin has clearly illustrated that plants cannot survive without N-glycans. For example, radish seeds are able to germinate on tunicamycin but young radish seedlings cannot survive more than 2 or 3 days after germination in the same culture conditions where N-glycosylation is prevented (Faye *et al.*, unpublished results). Tunicamycin is also highly toxic for mammalian-, yeast- or plant-cultured cells. However, by gradually increasing the concentration of the drug in the growth media, it was possible to select CHO cells [47, 92], as well as soybean cells [100] that can grow in the presence of tunicamycin at a concentration that is 100 times higher than the concentration of antibiotic sufficient to kill wild-type cells. As shown in the other systems able to survive in the presence of this glycosylation inhibitor, this is probably by over-expressing the target enzyme of tunicamycin, GlcNAc-1-phosphate transferase, that soybean cells could overcome the lethal inhibition of protein N-glycosylation. In contrast with the lethal effect of tunicamycin on unhabituated cells, it is possible to grow wild-type sycamore suspension-cultured cells in a medium containing castanospermine, a drug that prevents complex N-glycan maturation. In these conditions, sycamore cells are quite healthy for weeks despite that their glycoproteins have exclusively  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  N-glycans [48]. Does it mean that life for a plant is possible without complex-type N-glycans? So far, it seems to be the case. For instance, the results obtained using castanospermine are confirmed when the *cgl* mutant of *Arabidopsis thaliana* is considered. This mutant which lacks GNT I activity, the first glycosyltransferase in the pathway of complex-type N-glycan biosynthesis (Figure 7), is

completely unable to build complex-type N-glycans [91]. A CHO cell mutant lacking GNT I activity also does not synthesise complex-type N-glycans and is quite healthy [70, 71]. However, knocking out of the same enzyme is lethal in the early steps of mouse embryo development [38]. In contrast with its mammalian homologue, the *cgl* mutant of *Arabidopsis thaliana* is able to complete its development suggesting that complex-type N-glycans are not essential for normal developmental processes in plants. The *cgl* mutant should be a good model system for studying unknown biological functions of complex plant N-glycans. In this respect, A. Sturm and co-workers have compared the development of disease symptoms after infection by *Phytophthora parasitica* in wild-type *A. thaliana* and *cgl* mutant [46]. They found that the *cgl* mutant shows an enhanced susceptibility to compatible *Phytophthora parasitica* which could indicate that complex-type N-glycans may be involved in some defence reaction against infection.

New putative biological functions for plant N-glycans are now emerging since the recent identification of Lewis a ( $Le^a$ ) epitopes on plant N-linked carbohydrate. In mammals, Lewis antigens are usually found on glycoproteins or on cell-surface glycoconjugates and are known to be involved in a number of cell-cell recognition or adhesion processes [23]. For instance, the  $Le^a$  has been detected at the surface of cancer cells and could be involved in the process of metastasis [80]. So far, the biological relevance of such epitopes in plant glycoproteins is not known. However,  $Le^a$ -containing N-glycans were found attached to secreted proteins [26, 51] and to cell-surface proteins [26] suggesting a putative role in cell signalling. Particularly,  $Le^a$ -containing N-glycans are highly expressed at the plant cell surface and appear to be good candidates for cell communications or interactions with pathogens.

#### **Our current view of plant glycoproteins and human health: a little more than nutrients**

##### *Plant N-glycans and allergens*

The group of März [95] was the first to show that glycans N-linked to phospholipase A<sub>2</sub> (PLA<sub>2</sub>) are included in the IgE determinant of honey bee venom. In the same time, the immunogenicity of plant paucimannosidic-type N-glycans, their abundance in glycoproteins from *Dactylis glomerata* pollen and

their cross-reactivity with the oligosaccharide moiety from PLA<sub>2</sub> was described [20]. These observations have favoured the hypothesis that, in addition to the primary amino acid sequences and conformational structures of the protein backbone, specific carbohydrate structures constitutive of allergenic glycoproteins could also be involved in their reactivity with specific IgE antibodies. To better understand the involvement of N-linked oligosaccharides in various allergic reactions, several groups have looked at the carbohydrate structures of plant allergens. It has been shown that modified plant N-glycans are frequently observed on pollen grain and food allergens of plant origin. Furthermore, these oligosaccharides have been reported to be included in the IgE-determinant of these plant allergens [1, 29, 54, 55, 86, 87]. In most allergic reactions, patients have IgE antibodies directed to ubiquitous plant glycans, i.e. the  $\alpha(1,3)$ -fucose and/or the  $\beta(1,2)$ -xylose epitopes, leading to cross-reactivity between several foods and pollen allergens [1, 5, 20, 86], as well as cross-reactivity with insect allergens [1]. Hypersensitivity reactions related to food allergens of plant origin occurs in 6–8% of children. It is still a matter of debate whether or not IgE antibodies directed to N-glycans are biologically active, in other words, whether or not they induce clinical food allergy [88]. However, current research to overcome this problem include the development of foods without immunogenic N-glycans particularly through the transformation of the plant of interest by knocking out genes that encode enzymes responsible for the biosynthesis of the immunogenic carbohydrate epitopes.

A clear demonstration of the involvement of glycans N-linked to plant allergens in human allergies would have a very strong implication in the desensitisation of allergenic patients. Current immunotherapy uses crude allergen extracts injected to stimulate the production of IgG antibodies that play a role as blocking antibodies. However, in a near future, purified (natural or recombinant) allergens will be used for desensitization. If glycans are part of the IgE determinant, the recombinant allergens used for desensitization should present a similar glycosylation pattern as the natural allergen which will necessitate the use of transgenic plants as expression system for the production of recombinant plant allergens.

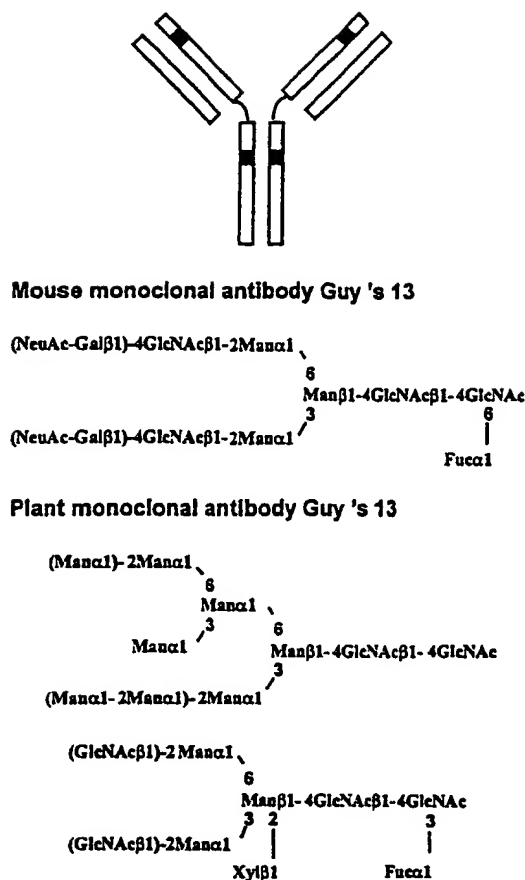


Figure 8. N-glycosylation of the mouse Guy's 13 monoclonal antibody produced in mouse and in transgenic tobacco plants. On the schematic representation of the structure of the IgG1 molecule, the two N-glycosylation sites are indicated in black on the heavy chain.

#### *Mammalian glycoproteins produced in transgenic plants have immunogenic glycans*

One of the most significant advance in DNA technology in the recent years is the development of recombinant protein expression in various systems. The ability of plants to express heterologous proteins has only been explored recently. To date, several N-glycosylated proteins from mammals have been expressed in transgenic plants [56]. As in other heterologous expression systems, glycosylation is one of the key steps in the production of functional glycoproteins in transgenic plants. N-glycosylation in higher organisms is conserved but differs in fine detail. The processing of the N-linked glycans occurs along the transport pathway as the glycoprotein moves from the

ER to its final destination through the Golgi apparatus. As developed previously in this review, glycosidases and glycosyltransferases located in the Golgi apparatus successively modify the oligosaccharide precursor to high-mannose-type N-glycans and then to complex-type N-glycans. Since some of these modifications are specific for the expression system, the structure of mature complex-type N-glycans associated with plant or mammalian glycoproteins differs. As mentioned earlier, plant complex-type N-glycans have an  $\alpha(1,3)$ -fucose residue on the proximal GlcNAc instead of a  $\alpha(1,6)$ -fucose residue in mammals, and a  $\beta(1,2)$ -xylose residue linked to the  $\beta$ -mannose. Since plants are gaining acceptance for the expression of recombinant therapeutic proteins, it is important to examine whether glycosylation will occur and which type of glycans will be present on recombinant mammalian glycoproteins produced in transgenic plants.

Immunoglobulins are good model glycoproteins for the evaluation of the potentiality of an expression system for the production of therapeutic glycoproteins. Indeed, it has been shown that some of the properties of immunoglobulins depend on its glycosylation. In general, there is one conserved N-glycosylation site per heavy chain of IgG in the  $CH_2$  domain constitutive of the Fc region of which the N-glycosylation is crucial to the structural stability of the immunoglobulin molecule. As a consequence, N-glycosylation is one of the key steps for the production of fully functional immunoglobulins by an heterologous expression system. The monoclonal antibody (MAb) Guy's 13 is a mouse IgG1 class antibody, which recognises a cell-surface protein of *Streptococcus mutans*, the bacterium that is the principal cause of dental caries in humans. This MAb contains two potential N-glycosylation sites on its constitutive heavy chain: the conserved glycosylation site in the Fc region and an additional site located in the Fab part of this IgG1 molecule. A full-length MAb Guy's 13 was expressed in tobacco [50]. This plant MAb was found to be functional in terms of antigen recognition and binding. The N-glycosylation of MAb Guy's 13 produced in mouse and in transgenic tobacco plants were recently compared [10]. N-glycosylation analysis of the mouse MAb Guy's 13 has shown that this IgG1 is N-glycosylated on both sites by biantennary N-glycans having  $\alpha(1,6)$ -fucose and about 10% of terminal sialic acid residues (Figure 8). The plant MAb Guy's 13 was also found to bear N-glycans on both sites of the heavy chains. A higher diversity was observed since both high-mannose-type and  $\beta(1,2)$ -xylose- and

$\alpha(1,3)$ -fucose-containing plant oligosaccharides were identified (Figure 8). Since the plant MAb Guy's 13 was found to be properly folded and functional [50], this result clearly illustrates that plants are able to introduce N-glycans on complex recombinant mammalian proteins in a sufficient way for a production of biologically active molecules. The complex glycans N-linked to the plant MAb Guy's 13 are not novel for humans who are exposed daily to such antigens in edible plant material. In this respect, the presence of these plant-specific oligosaccharides on the plant MAb Guy's 13 is not a limitation in the use of this antibody for oral applications to protect humans against dental caries. However, if people have prolonged exposure to large quantities of these highly immunogenic plant N-glycans as may be required by certain *in vivo* therapies, sensitisation to these specific plant antigens may occur.

As recently reviewed for insect cells [2], none of the transgenic host cell systems now available for the production of recombinant mammalian glycoproteins will produce these glycoproteins with the same glycans as normally found when they are produced in mammals. One of the advantages of plants as production systems for recombinant mammalian glycoproteins is our good knowledge of the protein N-glycosylation machinery and the availability of mutant plants allowing us to define strategies to produce recombinant proteins with more mammalian-like N-glycans. Two strategies to prevent the formation of highly immunogenic plant N-glycans on recombinant proteins emerge. The first possibility is to retain the recombinant glycoprotein in the ER so that the glycans added in this compartment will not be further modified in the Golgi apparatus into plant specific and immunogenic oligosaccharides. The second strategy, which allows the storage of the glycoproteins downstream from the Golgi apparatus, i.e. in the vacuole or in the extracellular compartment, is to modify the enzymatic machinery of the Golgi apparatus by knocking out enzymes (antisense strategy) and/or by adding new enzymes to modify the processing of N-glycans (transformation) (for a recent review on these strategies, see [13]).

## Conclusions

We are now beginning to understand the large capabilities of plant cells with respect to N-glycosylation. This is the result of intense efforts to improve analytical

procedures, together with a more precise knowledge of glycoprotein biosynthesis in the plant secretory pathway. Soon, cloning of glycosyltransferases will further highlight the specificities of glycosylation in the plant cell. The analysis of new glycosylation mutants and the knocking out of glycosyltransferases will help to better identify the biological functions of N-glycans in plants. Looking further into the future, it will make sense to engineer plant cells by knocking out part of their glycan processing machinery or by complementing this machinery with heterologous glycosyltransferases to produce recombinant glycoproteins with mammalian-like glycans at a lower cost than their mammalian counterparts.

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